Cloning and sequence analysis of cDNA encoding active phosphoenolpyruvate carboxylase of the C₄-pathway from maize

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ABSTRACT

A recombinant clone, pM52, containing cDNA for maize phosphoenolpyruvate carboxylase (PEPCase, EC 4.1.1.31) was isolated from a maize leaf cDNA library constructed using an expression vector in Escherichia coli. The screening of the clone was conveniently performed through its ability to complement the phenotype (glutamate requirement) of PEPCase-negative mutant of E. coli. The enzyme encoded by this clone was identical with the major PEPCase in maize, a key enzyme in the C₄-pathway, as judged from its allosteric properties and immunological reactivity. The cloned cDNA (3093 nucleotides in length) contained an open reading frame of 2805 nucleotides, the 3'-untranslated region of 222 nucleotides and the poly(dA) tract of 64 nucleotides. The deduced amino acid sequence (935 residues) of the enzyme showed higher homology with that of an enterobacterium, E. coli (43%) than that of a cyanobacterium (blue-green alga), Anacystis nidulans (33%).

INTRODUCTION

Phosphoenolpyruvate carboxylase (PEPCase) [EC 4.1.1.31] catalyzes CO₂-fixture on phosphoenolpyruvate (PEP) to form oxaloacetate and inorganic phosphate. The enzyme is widespread in higher plants, algae and many kinds of bacteria (1,2) and plays an anaplerotic role by replenishing C₄-dicarboxylic acid to the tricarboxylic acid cycle (3). Moreover, in C₄-plants such as maize and sugarcane, the enzyme plays a key role as a member of the C₄-pathway by which atmospheric CO₂ is primarily trapped and transferred to the Calvin cycle (4). PEPCase is now regarded as one of important targets in plant breeding by genetic manipulation, since the increment of PEPCase together with other enzymes in C₄-pathway is expected to bring about an enhancement of photosynthetic CO₂-fixture ability in plants (5).

PEPCases from various sources are usually active as tetramers and their subunit molecular weights (MW) range from 90,000 to 100,000 (see refs. 6 and 7 for the maize and E. coli enzymes, respectively). Reflecting the central role of this enzyme in cellular metabolism, most of the enzymes hitherto investigated are allosteric in nature, though their effector compounds differ widely.
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depending on the source of enzyme (see refs. 6 and 8 for the maize enzyme and ref. 9 for the E. coli enzyme).

We have been studying the structure of PEPCase genes of several organisms to elucidate the molecular basis for the effector diversity and to pursue the possibility of manipulating the PEPCase level in photosynthetic organisms by genetic engineering. Previously, we cloned the genes for PEPCase (ppc) from E. coli (10,11) and a cyanobacterium (blue-green alga), Anacystis nidulans (12), and determined their nucleotide sequences (13,14). We report here the cloning and sequence analysis of a maize cDNA which encodes active PEPCase in E. coli cells. To facilitate the screening for PEPCase cDNA, a cDNA library for maize mRNA was constructed by using a plasmid vector specially designed for cDNA expression in E. coli, and search was made for clones which correct defect of an E. coli ppc mutant. Furthermore, the deduced amino acid sequence of maize PEPCase was compared with those of E. coli and A. nidulans. So far as we know, this is the first report on the cloning and sequence analysis of cDNA encoding active PEPCase from higher plants.

MATERIALS AND METHODS

Materials

Reverse transcriptase (RNA-dependent DNA polymerase) from Avian myeloblastosis virus was obtained from Seikagaku Kogyo Co. (Japan). Restriction enzymes and other DNA modifying enzymes were purchased from PL-Biochemicals Inc. (USA), Takara Shuzo Co. (Japan), and New England Biolabs (USA). The M13 sequencing Kit and [α-32P]dCTP (>400 Ci/mmol) were obtained from Takara Shuzo Co. and Amersham (UK), respectively. Oligo(dT)-cellulose (Type 7) and horseradish peroxidase-conjugated goat anti-rabbit IgG were from PL-Biochemicals Inc. Crude preparation of PEPCase from maize leaves and a rabbit antiserum against this enzyme were prepared as described in refs. 6 and 15, respectively.

Bacterial strains and plasmids

The following strains of E. coli K-12 were used: W3110 (10), DH1 (16), PCR1 (11) and JM101 (17). Plasmids used were pM52 isolated in this study, pS3 carrying the E. coli ppc gene (11) and pSI4001 (S. Itamura and K. Shigesada, to be published elsewhere). Plasmid pSI4001 (4 kilobase pairs, kb, in size) was constructed so as to permit expression of a cDNA insert in any reading frame in E. coli cells. The cloning vector system developed by the use of pSI4001 was similar to that by Okayama and Berg (18), but different from it in carrying the following segments instead of the segment from SV40. Essential-
ly, the HindIII/PstI segment (0.38 kb) adjoining the 5' end of the cDNA insert contained the lac promoter, the start codon of lacZ and multiple cloning sites (EcoRI/BamHI/SalI/PstI) which had been adapted from M13mp7 (17). In addition, a synthetic DNA oligomer containing a ribosome-binding site and an ATG codon (GAGGAAACAGCTATG) was inserted tandemly at the EcoRI/BamHI and BamHI/SalI junctions so as to provide the vector with three potential translation start sites of mutually distinct reading frames including the one carried over from M13mp7 mentioned above.

**Growth of bacteria**

E. coli cells were grown in Luria broth (LB medium, cf. 10) or a synthetic medium (glycerol-salts medium) which consisted of medium E (cf. 10), glycerol (0.5%), isopropyl-β-D-thiogalactopyranoside (2x10⁻⁵ M), and Anraku's trace-metal solution (1 ml/l)(cf. 10). The media were supplemented with appropriate antibiotic(s) and amino acids when necessary (12).

**Preparation of poly(A)⁺ RNA from maize leaves**

Seeds of Zea mays L. var. indentata STURT. were placed on wet paper towels, germinated and grown in a 28°C chamber. The leaves were harvested from plants grown for 10 days in total darkness plus under constant illumination (1x10⁶ lux) for 3 days. Total RNA (1.8 mg) was obtained from 3.8 g wet leaves by the method of Sims and Hague (19), and poly(A)⁺ RNA (3.5 μg) was isolated by oligo(dT)-cellulose chromatography (20).

**Construction of cDNA library**

Poly(A)⁺ RNA was cloned essentially as described by Okayama and Berg (18) by the use of pSI4001 as a vector. The cloned circular DNA formed was transfected into E. coli DH1 according to the protocol of Maniatis et al. (21). Ampicillin-resistant transformed cells were pooled, added to 500 ml of LB medium containing 100 μg of ampicillin per ml, and grown at 37°C for 24 hr. A hundred ml of the stationary culture was adjusted to 7% dimethylsulfoxide and stored in 2-ml portions at -70°C. From the rest of the culture plasmid DNAs were prepared as described (12). The cDNA library which was obtained from 3.5 μg of poly(A)⁺ RNA consisted of 6 X 10⁵ independent clones.

**Primary screening for the cDNA clone encoding PEPCase**

To isolate cDNA clones encoding PEPCase, the cDNA library was screened by the phenotypic complementation test as described previously (12). E. coli PCR1 which carries ppc and recA1 mutations (11) was first transformed with the cDNA library and plated on solidified LB medium containing ampicillin (50 μg/ml). Ampicillin-resistant clones were replica-plated on the glycerol-salts medium supplemented with or without L-glutamate (1 mg/ml) and grown at 30°C. Search
was made for the clone which was able to grow without glutamate supplement.

**Assay of PEPCase**

The preparation of cell extracts, the assay of PEPCase and determination of protein were carried out as described (12). One unit of the enzyme was defined as the activity oxidizing 1 μmol of NADH/min at 30°C. For the detection of PEPCase in the polyacrylamide gels after electrophoresis, the gels were immersed in the reaction mixture for PEPCase at 30°C for 30 min, and oxaloacetate formed in the gels was visualized with Fast-Violet B (22). The reaction mixture contained 2 mM PEP, 10 mM KHCO₃, 10 mM MgSO₄, 590 mM dioxane (an activator of *E. coli* PEPCase) and 100 mM Tris- H₂SO₄ buffer (pH 8.5).

**Nucleotide sequence analysis**

Nucleotide sequence analysis was performed using the enzymatic chain termination method (23) in conjunction with M13 derived vectors (mp18 and mp19) (24). The DNA fragments to be sequenced were generated either by restriction enzymes or by sonication. The sonication was carried out as described by Deininger (25) with the NruI/PvuI fragment (3.1 kb, 5 μg) and the generated fragments with a size range of 500-1000 bp were inserted into the M13mp19 SmaI site, after enzymatic repair of their ends.

**RESULTS**

**Isolation of PEPCase cDNA clone**

Total RNA was prepared from young leaves of *Zea mays* harvested after 3 days of constant illumination, since greening of etiolated tissues is known to result in a pronounced increase in mRNA for PEPCase (19). Poly(A)⁺ RNA was cloned essentially by the method of Okayama and Berg (18), except that a new expression vector, pSI4001 was used instead of pBR-SV40 chimeric plasmids originally described. The cDNA library was subjected to the screening by transformation of *E. coli* PCR1 cells, which require glutamate for growth on sugar or glycerol as a carbon source owing to the defect in ppc gene (3). The ampicillin-resistant transformants initially grown on LB medium were replicated on the selective medium as described in MATERIALS AND METHODS. One transformant which was able to grow without glutamate was obtained among 8400 transformants and was found to carry a plasmid (named pM52) containing a cDNA insert of about 3 kb. The isolated plasmid pM52 DNA was able to transform PCR1 to ampicillin resistance with an efficiency of 10⁶ transformants per μg DNA, and all the transformants grew in the absence of glutamate. Thus, it is clear that this plasmid by itself bears the ppc-complementing activity.
Table 1. Characterization of PEPCase activity in E. coli cells (PCR1) transformed with pM52.

<table>
<thead>
<tr>
<th>Additions</th>
<th>PCR1/pM52</th>
<th>PCR1/pS3</th>
<th>W3110</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>7.0</td>
<td>15</td>
<td>0.36</td>
</tr>
<tr>
<td>L-Malate (10 mM)</td>
<td>5.3</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>L-Aspartate (10 mM)</td>
<td>6.7</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Acetyl-CoA (0.093 mM)</td>
<td>7.9</td>
<td>267</td>
<td>11.2</td>
</tr>
<tr>
<td>Glycine (10 mM)</td>
<td>9.1</td>
<td>11.7</td>
<td>0.12</td>
</tr>
<tr>
<td>Glucose 6-phosphate (10 mM)</td>
<td>19.3</td>
<td>14.7</td>
<td>0.40</td>
</tr>
<tr>
<td>Dioxane (1.17 M)</td>
<td>2.9</td>
<td>582</td>
<td>17.0</td>
</tr>
</tbody>
</table>

PEPCase activity was determined with cell extracts of the transformants PCR1/pM52 and PCR1/pS3 which carries a plasmid (pS3) containing the E. coli ppc gene, and also with cell extract of the wild-type strain of E. coli K-12 (W3110). From the cells of three strains grown in a glycerol-salts medium, the cell extracts were prepared and assayed for PEPCase activities as described in MATERIALS AND METHODS. For characterization of PEPCase of PCR1/pM52, various compounds known as effectors for maize (6,8) and E. coli (9) PEPCases were tested at the concentrations indicated. No activity was detected with the extracts from PCR1.

Characterization of the cDNA-encoded enzyme

Cell extracts from the transformants and other E. coli strains were assayed for PEPCase activity. Although there was no enzyme activity in the extracts from the original host strain, PCR1 (data not shown), significant activities were found in all of the extracts from randomly selected clones of PCR1 transformed with pM52. Typical data are shown in Table I. The specific activity detected in PCR1/pM52 without any allosteric effector was about 20-fold higher than that in E. coli W3110 (wild-type strain) and about a half that in E. coli PCR1 transformed with a plasmid (pS3) containing the E. coli ppc gene (11).

Zea mays and E. coli PEPCases are both allosterically regulated, but they respond to different effectors. The enzyme activities in PCR1/pS3 and W3110 were inhibited by L-malate and L-aspartate and activated by acetyl-CoA and dioxane (a non-physiological organic solvent) as expected for E. coli PEPCase. In contrast, the enzyme activity in PCR1/pM52 showed a slight inhibition by L-malate and a significant activation by glycine and glucose 6-phosphate (6), but it was activated neither by acetyl-CoA nor by dioxane. Although no significant inhibition by L-aspartate was observed against expectation, this might be due to suboptimal assay conditions used where the Mg$^{2+}$ concentration (10 mM) and pH (=8.0) were too high (8). These data indicate that the enzyme in
Fig. 1  Electrophoresis of cell extracts from PCR1/pM52 on polyacrylamide gels containing (b) and not containing (a) SDS.  a. Localization of PEPCase activity.  Cell extracts prepared as in Table 1 were subjected to an electrophoresis on polyacrylamide gels (7.5 %, pH 8.9) as described (10).  After electrophoresis the gels were stained for PEPCase as described in MATERIALS AND METHODS.  Lane 1, PCR1/pS3; lane 2, W3110; lane 3, PCR1; lane 4, PCR1/pM52.  b. Western blotting analysis (26) using antiserum to maize PEPCase.  Cell extracts and the extracts from maize leaves were electrophoresed in 10 % polyacrylamide gels containing 0.1 % SDS (27).  After electrophoretic blotting transfer to Durapore membrane (GVHP, Millipore), reactive proteins were visualized with a rabbit antiserum to maize PEPCase and horseradish peroxidase-conjugated goat anti-rabbit IgG, followed by color development with 4-chloro-1-naphthol.  Lane 1, PCR1/pM52; lane 2, PCR1/pS3; lane 3, extracts from maize leaves; lane 4, PCR1.

PCR1/pM52 has allosteric properties characteristic of the \( Z. \) mays enzyme involved in the \( C_4 \)-pathway (6).

Electrophoresis of cell extracts on polyacrylamide gels accompanied by staining for PEPCase activity revealed a single activity band for each cell source.  The enzyme from PCR1/pM52 (Fig. 1a, lane 4) moved toward anode faster than those from PCR1/pS3 (lane 1) and W3110 (lane 2).  Moreover, the pM52-encoded enzyme gave a rather diffuse band, indicating some microheterogeneity, whereas the \( E. \) coli enzyme gave a sharp activity band.  To confirm further that the PEPCase activity expressed in PCR1/pM52 is of maize origin and to
Fig. 2 Diagram of the structure of maize PEPCase cDNA clone (pM52) and the strategy for DNA sequence analysis. Plasmid pM52 consisted of the DNA segment (3.7 kb) (shown) and the HindIII/PvuII segment (2.3 kb) of pBR322 containing ori- and amp\textsuperscript{r} regions (not shown) (28). The thick lines on both sides represent the segments originated from the vector plasmid (pSI4001). The hatched box depicts the poly(dG)\textsuperscript{r}poly(dC) homopolymeric extension used for ligation of cDNA to the PstI-cleaved end of the vector-primer and the open box the cloned cDNA insert, which includes the coding region for PEPCase, 3'-untranslated region and poly(dA)\textsuperscript{r}poly(dT) tract. The restriction map indicates sites for 6-bp cutters within the insert. The direction and extent of sequence determinations are shown by horizontal arrows. The arrows with closed circle at one end indicate sonication-generated fragments and the arrows with vertical bar, restriction enzyme-generated fragments.

estimate the MW of its subunit polypeptide, the cell extracts were subjected to Western blotting analysis (26) after electrophoresis on polyacrylamide gels containing sodium dodecyl sulfate (SDS). Figure 1b shows that a polypeptide reacting with a rabbit antibody raised against maize PEPCase, was specifically detected in the extracts from PCR1/pM52 (lane 1). The MW of the polypeptide was estimated to be about 100,000, being almost equal to that of PEPCase prepared from maize leaves (lane 3). These results demonstrate that the isolated clone (pM52) does contain a cDNA for maize PEPCase, which is long enough to encode an active enzyme protein with known allosteric properties.
ATG GAC CGG ATC AAG GCC AAG CAG CAG TGG CAT CAG TTC ATG GAC CAA GTG ACT CAG GTC ATG GTC GGC TAC TCC GAC TCC GGC AAG GAC GCC GGC CGC 1725
Met Asp Arg Ile Lys Gly Lys Gin Gin Val Met Val Gly Tyr Ser Asp Ser Gly Lys Asp Ala Gly Arg 575
CTG TCC GGC GGG TGG CAG CTG TAC ARG AAG GCC AAG CAG CAG GTT CAG GAC AGT GGC CAG GCC GGC CAG GTC GTC GGC TAC TCC GAC TCC GGC AAG GAC GCC GGC CGC 1794
Leu Ser Ala Ala Trp Gin Leu Tyr Arg Arg Gin Gin Val Met Met Gin Met Ala Val Lys Arg Tyr Gly Val 598
AAG CTC ACC TTG TTC CAC GCC GGC GAA GGC ACC ATG GAC GGC GGG CAC ACC TTG TGT GTC GTC GTC AAG GAG CCGCGTCC GGATGGTGGCCGTTTTGTCAGTTTTGGATGGAAATGCCGGAACCTGGCCAGCGTCTGTTTTCCCTATGCATATGTAATTTCCTGCCTCTTTATATTCACTCTTGTTGTCAAGTCCAAGTGGAAAATCTTGGCATATTATACATATTGTAATAATAAGCATCG
TACAATCTGC(A) 54

Fig. 3 Nucleotide sequence of cDNA and deduced amino acid sequence for *Zea mays* PEPCase subunit. The number at the end of each line indicates the position of the final residue (nucleotide or amino acid) on that line. The first codon within the cDNA insert was AGU, so that the A residue at the first position of this codon is numbered as 1, and the deduced amino acid residue, Ser, was numbered as 1. The overlined sequence from -78 to -3 indicates the 5'-flanking sequence from the second translation initiation module of the vector and the poly(dG) tract used for ligation. The amino acid sequence from -21 to -1 coded by this sequence is presumably appended to the sequence coded by cDNA insert. The putative overlapping polyadenylation signal (29) is underlined.
The restriction map of the cDNA insert in pM52 was constructed by standard procedures, and the DNA sequence was determined according to the strategy shown in Fig. 2. The entire nucleotide sequence of the 3093 bp insert is shown in Fig. 3. The insert contained a long open reading frame of 2805 nucleotides which corresponds to 935 amino acid residues. The codon specifying the glycine residue at position 2805 was followed by the translation termination codon UAG. The length of the 3'-untranslated region was 222 nucleotides excluding the poly(dA) tract consisting of 64 nucleotides. Two overlapping copies of polyadenylation signal (29), AATAATAA, were found 16 nucleotides upstream from the poly(dA) tract. The 5'-end of the cloned coding region was directly flanked by a poly(dG) tract of 26 bp, indicating that the cloned cDNA lacked the 5'-untranslated region and coding region for the N-terminal of the original mRNA. Thus the gene appears to be expressed in E. coli cells as a fusion protein with an N-terminal appendage encoded by the second initiation module, the poly(dG) tract and the 5'-terminal two nucleotides (CG) of the cDNA insert. The nucleotide sequence and the deduced amino acid sequence of the N-terminal appendage are indicated by an overline in Fig. 3. The calculated MW of PEPCase subunit polypeptide encoded by the cloned cDNA was 105,540. Since this value was even larger than that (100,000) estimated for the enzyme by SDS-polyacrylamide gel electrophoresis (6), the missing N-terminal portion of native PEPCase must be only minimal. Comparison of the codon usage within the cDNA insert with those in the ppc genes from E. coli and A. nidulans (data not shown, cf. refs. 13 & 14) shows a marked preference for codons with C or G at the third position among synonymous pairs. In a total number of 902 codons (excluding codons for Met and Trp having only one set of codon), the codons which have A, U, G and C at the third position were 68, 88, 328 and 418, respectively. This codon usage was similar to but more biased than that in the alcohol dehydrogenase (Adhl) gene of maize (30).

Amino acid sequence homology between PEPCases from maize, enterobacterium and cyanobacterium

We compared the deduced amino acid sequence of Zea mays PEPCase with those of E. coli and A. nidulans PEPCases reported previously. The sequence of the E. coli enzyme was deduced from the nucleotide sequence and was confirmed by protein-chemical methods (13). The sequence of the A. nidulans enzyme, however, was the one deduced from the open reading frame (14), which has not yet been confirmed protein-chemically. Figure 4 shows the three
Fig. 4 Comparison of amino acid sequence of Zea mays PEPCase (Z) with those of Anacystis nidulans (A) (14) and Escherichia coli PEPCases (E) (13). Alignments were made by visual inspection. The one-letter amino acid notation is used, and the numbers of the residues on each line are given on the left side. The identical residues between Z. mays and A. nidulans enzymes and between Z. mays and E. coli enzymes are indicated by asterisks. Gaps, attributed to deletions or insertions, appear as blank spaces.
sequences aligned together to maximum homology. Although the numbers of amino acid residues of these three polypeptides are considerably different from each other (935, 1053 and 883 residues for *Z. mays*, *A. nidulans* and *E. coli* enzymes, respectively), their C-terminal sequences are conserved strikingly well. The size difference is largely attributable to variations in the length of N-terminal extensions. When the overall homology between each pair of peptides (or segments of peptides) is represented by proportion (%) of exact matches of amino acid residues in the total number of residues of the shorter peptide, they were 33% between the *Z. mays* and the *A. nidulans* enzymes, 43% between the *Z. mays* and the *E. coli* enzymes, and 35% between the *E. coli* and the *A. nidulans* enzymes. Several regions of particularly high homology (>50%) common to all three polypeptides were found by visual inspection as follows: residues 137-165, residues 250-269, residues 401-467, residues 497-519, residues 560-642, residues 681-762 and residues 922-935 (Numbers of residue refer to the sequence of *Z. mays* PEPCase).

**DISCUSSION**

In this work, a cDNA clone encoding active PEPCase was conveniently screened through its ability to complement the defect of an *E. coli ppc* strain. This exemplified the general utility of *E. coli* mutants defective in particular gene in isolation of the corresponding plant or mammalian genes, as suggested by Davidson & Niswander (31).

Plasmid pM52 seems to contain near full-length cDNA for PEPCase but devoid of the 5'-terminal sequence of mRNA including the start codon and upstream untranslated region. We are now trying to clone the full length cDNA. Since the elucidation of intron-exon structure should provide useful information on the domain structure of the enzyme protein (32), we are also trying to clone the genomic DNA for PEPCase from maize.

We have detected significant amino acid sequence homologies among three PEPCases from *Z. mays*, *A. nidulans* and *E. coli*. It is of interest to note that the homology between *Z. mays* and *E. coli* enzymes is higher than that between *Z. mays* and *A. nidulans* enzymes. Although there are a number of stretches of conserved sequences throughout the entire length of the polypeptide, they are notably more concentrated in the C-terminal halves. Namely, the region corresponding to the residues 401-826 in *Z. mays* cDNA contained 41 and 51% exact matches with the *A. nidulans* and *E. coli* enzymes, respectively. Such a strong sequence conservation among proteins from phylogenically distant species may be taken to reflect functional constraint on the encoded product.
We therefore propose that the conserved region in PEPCase may comprise part of the catalytic site. As already noted, the sequences of the C-terminal portion (10 residues) are also highly conserved. We observed previously that the plasmid pS2ΔB, which codes for E. coli PEPCase with a deletion in the C-terminal 22 amino acids, was unable to complement the ppc mutation, and that only a small amount of polypeptide encoded by the plasmid was accumulated in the E. coli cells (11). Accordingly this portion seems to be indispensable for the formation of active enzyme and/or the in vivo stability of the enzyme.

The sequences of N-terminal halves are considerably different from each other except for a few stretches of highly conserved sequences. The less-conserved region may reflect the differences in regulatory properties of the three enzymes: The enzymes from Z. mays and E. coli are allosteric in nature though their effectors are different, while the enzyme from A. nidulans seems to have no effectors so far as investigated (Kuriyama, M., Hirai, K., Izui, K. & Katsuki, H., unpublished data). Thus it is tempting to speculate that the domains formed by N-terminal halves are involved in the allosteric regulation. Attempts are being made to modify the allosteric properties of PEPCase by making chimeric enzymes in which C- and N-terminal halves of the enzymes from different organisms are exchanged with each other.

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